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# Outbreak of *Citrobacter freundii* carrying VIM-1 in an Italian Hospital, identified during the carbapenemases screening actions, June 2012

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SUMMARY

*Objective:* The identification of patients colonized or infected with carbapenemase-producing *Enterobacteriaceae* (CPE), in order to control and prevent the global spread of multidrug-resistant (MDR) pathogens.
*Methods:* From June 1 to June 15, 2012, eight *Citrobacter freundii* strains with reduced susceptibility to carbapenems were isolated from rectal swabs of hospitalized patients during active screening following the detection of a Klebsiella pneumoniae carbapenemase (KPC) -positive patient on the ward. All isolates were analyzed phenotypically and molecularly by PCR and sequencing. Genotype clustering was performed by multilocus sequence typing (MLST) analysis.
*Results:* The isolates showed high rates of multidrug resistance profile. A phenotypic assay for carbapenemase production suggested the presence of metallo-β-lactamase (MBL). The *blaVIM-1* gene was detected in all imipenem-resistant *C. freundii* isolates. MLST showed that the *C. freundii* isolates shared the same sequence type (ST). Phylogenetic analysis revealed a strict relationship with an ST5 *C. freundii* isolate from a diarrhea patient in China.
*Conclusions:* Our findings showed that the active surveillance program for CPE was useful, not only for the detection of KPC-producers, but also to identify and control the spread of other MDR pathogens that could expand the spectrum of circulating MDR pathogens.


## 1. Introduction

In recent years, the emergence and the very rapid spread of *Enterobacteriaceae* carrying carbapenem-hydrolyzing lactamases (carbapenemases) have been observed throughout the world, representing a serious threat to public health due to the great limitations of antimicrobial therapy.[1] Recently, several outbreaks of plasmid-acquired carbapenemase-producing *Enterobacteriaceae* (CPE) have been reported in Europe, suggesting an intense circulation of these microorganisms in this area.[1] Among the different types of carbapenemases, class A klebsiella pneumoniae carbapenemase (KPC) and class B (Verona integron-encoded metallo-b-lactamase (VIM), Imipenemase (IMP), New Delhi metallo-β-lactamase (NDM)) carbapenemases have represented the most common findings, while more recently class D oxacillinase-48 (OXA-48) has emerged.[1] In particular, Italy and Greece have reported a great spread of KPC and metallo-β-lactamases (MBL), mostly of VIM and NDM.[1–6]

Carbapenemase-producers are mainly identified among *Klebsiella pneumoniae* and *Escherichia coli*, while for other *Enterobacteriaceae* this type of resistance is still relatively uncommon.[1] Only a few studies have reported *Citrobacter freundii* harboring MBL or KPC carbapenemases, most of them carrying *blaVIM* or *blaKPC* genes.[5–7] Moreover, the isolation of a VIM-producing *C. freundii* is still rare in Europe.[6,7] Up until today, only one study has reported the finding of an MBL-producing *C. freundii* isolate in Italy.[8] Here, we describe an outbreak of *blaVIM-1*-producing *C. freundii* isolated from rectal swabs of colonized patients hospitalized in an Italian hospital.

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## 2. Methods

### 2.1. Antimicrobial susceptibility testing and molecular analysis of C. freundii isolates

From June 1 to June 15, 2012, eight isolates of *C. freundii* were isolated from rectal swabs of patients hospitalized on a medical ward of a hospital located in the Bologna metropolitan area as a result of active screening following the detection of a KPC-positive patient on the same ward. Each *C. freundii* strain was isolated using a chromogenic selective medium (Brilliance CRE, Oxoid, UK). Routine biochemical identification and antimicrobial susceptibility testing were carried out using the Vitek2 semi-automated system (bioMérieux, France). Minimum inhibitory concentration (MIC) results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.[9] Carbapenem MIC values were confirmed by E-test. *C. freundii* isolates were further tested by a disk-diffusion synergy test as confirmatory/discriminatory carbapenemase phenotypic assay.[10] A PCR assay for *bla* genes, including *VIM*, *IMP*, *GIM*, *SIM*, and *SPM* was performed in order to identify the specific MBL gene, and the amplicons obtained were analyzed in order to identify the specific *bla* gene.[11,12] Results were compared and aligned with reference sequences using the online BLAST database and CLUSTAL W software.

### 2.2. Multilocus sequence typing (MLST)

To determine the genetic relationships between the *C. freundii* isolates, MLST based on seven housekeeping genes (*aspC, clpX, fadD, mdh, arcA, dnaG, lypS*) was performed, as described previously.[13]

### 2.3. Cluster management and infection control

In July 2011, the health agency of the Emilia-Romagna region issued a specific surveillance protocol in order to improve the prevention and control of CPE.[14] The implementation of the regional guidelines by local hospitals requires extended infection control measures, including: isolation or cohorting of colonized/infected patients; strict contact precautions and reinforced hand hygiene; active surveillance by rectal swab cultures; screening of contact patients (when a positive case is detected); and, for patients with higher clinical and/or epidemiological risks, culture screening upon admission to the hospital.

## 3. Results

From June 1 to 15, 2012, eight *C. freundii* isolates were recovered from specimens of colonized patients. The isolates were resistant to all β-lactams, β-lactam/β-lactamase inhibitor combinations, and imipenem, while still susceptible to ciprofloxacin, colistin, tigecycline, trimethoprim–sulfamethoxazole, meropenem, and ertapenem (Table 1). The synergy test gave positive results for the meropenem disk in association with dipicolinic acid for all eight strains, suggesting the production of MBL. PCR analysis and nucleotide sequencing revealed that all these isolates carried the *blaVIM* gene. Sequence analysis of the eight cloned amplicons performed with BLAST software showed 100% homology with the *blaVIM-1* sequences available in GenBank.

The MLST assay revealed that all the *C. freundii* strains had identical allele profiles. Comparison of the seven housekeeping genes obtained from a previously described human *C. freundii* isolate in China revealed several nucleotide substitutions among the seven housekeeping genes (data not shown). In particular, the highest variability was identified in the *fadD* gene (which is known to be the most variable among these seven genes). In order to evaluate the genetic relatedness of these *C. freundii* strains with previously reported isolates, we inferred an evolutionary relationship by constructing a neighbor-joining tree using a concatenated sequence of the seven housekeeping genes obtained from different human *C. freundii* isolates, as shown in Figure 1. The tree showed strict clustering of the Italian *C. freundii* isolates with the ST5 isolated in China from a patient suffering from acute diarrhea.[13]

On June 1, 2012, the local infection control committee of one of the hospitals located in the Bologna metropolitan area, received information about the isolation of a KPC-positive *K. pneumoniae* strain from a patient who was hospitalized on a medical ward. The committee correctly suggested that the affected ward apply active surveillance for all potential contact patients. As a consequence, 42 rectal swabs were taken within 1 day. This screening activity showed the detection of four patients colonized with a VIM-

**Table 1**
Results of antimicrobial susceptibility testing of VIM-1-producing *Citrobacter freundii* strains: MIC values (mg/l) and EUCAST susceptibility interpretation[a]

| No. of isolate | Patient sex and age, sample collection date (day/month/year) | Antimicrobial | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AMC | TZP | CTX | CAZ | ERT | IPM | MEM | AK | GM | CIP | TIG | COL | SXT |
| CF1 | F, 90 | ≥32 | ≥128 | ≥64 | ≥64 | 0.5 | >32 | 2 | 16 | ≥16 | ≤0.25 | ≤0.5 | ≤0.5 | ≤20 |
| | (01/06/12) | R | R | R | R | S | R | S | I | R | S | S | S | S |
| CF2 | M, 87 | ≥32 | ≥128 | ≥64 | ≥64 | 1 | >32 | 2 | 16 | ≥16 | ≤0.25 | ≤0.5 | ≤0.5 | ≤20 |
| | (01/06/12) | R | R | R | R | I | R | S | I | R | S | S | S | S |
| CF3 | F, 93 | ≥32 | ≥128 | ≥64 | ≥64 | 0.5 | >32 | 1 | 32 | ≥16 | ≤0.25 | ≤0.5 | ≤0.5 | ≤20 |
| | (01/06/12) | R | R | R | R | S | R | S | R | R | S | S | S | S |
| CF4 | F, 74 | ≥32 | ≥128 | ≥64 | ≥64 | 0.5 | >32 | 1 | 16 | ≥16 | ≤0.25 | ≤0.5 | ≤0.5 | ≤20 |
| | (01/06/12) | R | R | R | R | S | R | S | I | R | S | S | S | S |
| CF5 | M, 97 | ≥32 | ≥128 | ≥64 | ≥64 | 0.5 | >32 | 1 | 16 | ≥16 | ≤0.25 | ≤0.5 | ≤0.5 | ≤20 |
| | (08/06/12) | R | R | R | R | S | R | S | I | R | S | S | S | S |
| CF6 | F, 83 | ≥32 | ≥128 | ≥64 | ≥64 | 0.5 | >32 | 1 | 16 | ≥16 | 0.5 | ≤0.5 | ≤0.5 | ≤20 |
| | (08/06/12) | R | R | R | R | S | R | S | I | R | S | S | S | S |
| CF7 | M, 76 | ≥32 | ≥128 | ≥64 | ≥64 | 0.5 | >32 | 1 | 16 | ≥16 | 0.5 | ≤0.5 | ≤0.5 | ≤20 |
| | (15/06/12) | R | R | R | R | S | R | S | I | R | S | S | S | S |
| CF8 | M, 74 | ≥32 | ≥128 | ≥64 | ≥64 | 0.5 | >32 | 1 | 16 | ≥16 | ≤0.25 | ≤0.5 | ≤0.5 | ≤20 |
| | (15/06/12) | R | R | R | R | S | R | S | I | R | S | S | S | S |

MIC, minimum inhibitory concentration; EUCAST, European Committee on Antimicrobial Susceptibility Testing; AMC, amoxicillin–clavulanic acid; TZP, piperacillin–tazobactam; CTX, cefotaxime; CAZ, ceftazidime; ERT, ertapenem; IPM, imipenem; MEM, meropenem; AK, amikacin; GM, gentamicin; CIP, ciprofloxacin; TIG, tigecycline; COL, colistin; SXT, trimethoprim–sulfamethoxazole.

[a] Interpretation: S = susceptible; I = intermediate; R = resistant.

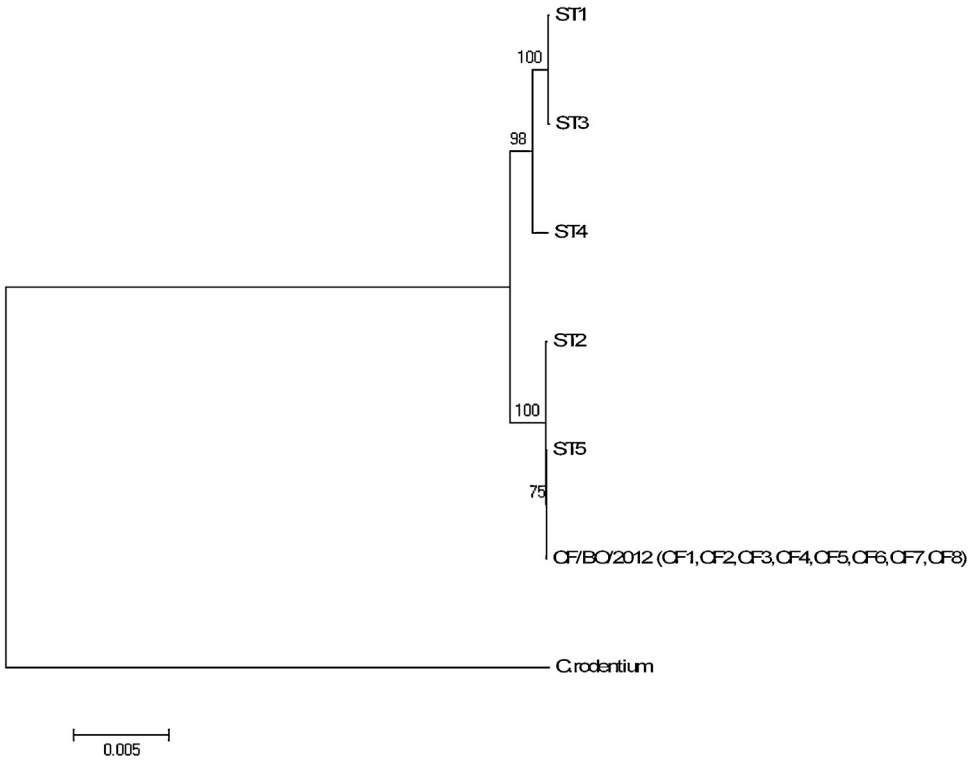


**Figure 1.** Genetic relationships among *Citrobacter freundii* strains isolated from patients in Italy and in China,[13] as indicated by multilocus sequence typing data. The phylogenetic tree was inferred using the neighbor-joining (NJ) method based on the aligned concatenated sequences of the seven housekeeping genes obtained from the different sequence types (ST) and *Citrobacter rodentium* (used as the outgroup).

producing *C. freundii*, while no other KPC-*K. pneumoniae* were found. During the following 2 weeks, four other patients from the same ward had a positive result for *C. freundii*, showing an identical phenotype. No clinically relevant isolates of these microorganisms were found among the patients hospitalized on the affected ward. Even though a detailed epidemiological investigation was performed, the evaluation of collected data did not permit the identification of the potential index case.

The cluster of colonized patients was managed by applying the measures suggested by the regional protocol: infection control operators visited the ward to investigate any incorrect care behavior and meetings to refresh and improve staff training were organized; all the colonized patients were subjected to cohorting to ensure the strict application of contact precautions; microbiological screening was performed to monitor the transmission of CPE to other patients. This early and intensive action allowed containment of the cluster and no more cases were detected starting from June 16, 2012. The screening activity was continued for an additional 4 weeks, and no more *C. freundii* bearing *blaVIM-1* were identified.

## 4. Discussion

The global spread of *Enterobacteriaceae* carrying plasmid-mediated carbapenem resistance has been observed worldwide, but since 2010 the increase in these MDR organisms has been very rapid, especially in Europe.[1] Based on previous reports, the epidemiology of *Enterobacteriaceae* harboring different carbapenemase types, such as NDM and KPC, suggests a condition of endemicity in the Italian area.[1–4] Among the different carbapenemase-producing *Enterobacteriaceae*, the most common species is *K. pneumoniae*, followed by *E. coli*, while various others species have been isolated sporadically both from infected and colonized patients.[1] In particular, few studies have reported on the isolation of *C. freundii* strains carrying carbapenemase genes.[5,6,8] This study reports an outbreak of VIM-producing *C. freundii* in an Italian hospital. MLST analysis grouped all these eight *C. freundii* isolates in a close relationship with the ST5 strain recently reported from a diarrhea patient in China.[13]

The rapid identification of patients colonized and infected with CPE is recognized as a basic tool to control and prevent the global spread of these MDR pathogens.[1] In our study, we showed that active surveillance performed by rectal swab culture screening of all the potential contacts is useful, not only to control inter-patient transmission of a previously known MDR strain, but also to detect additional colonization with other types of CPE with the potential to spread among hospitalized patients. This approach thus represents an efficient measure to minimize the risk of nosocomial outbreaks caused by carbapenemase producers. The accurate and rapid identification of VIM-producing *C. freundii* strains allowed the infection control team to rapidly detect a cluster of patients colonized with this unusual type of CPE. This quick identification allowed the medical ward to contain the dissemination of these MDR bacteria among the hospitalized patients. It is noteworthy that despite the unfavorable clinical features of most patients (mean age >84 years, many comorbidities), colonization had not given rise to any clinically significant infection. This favorable clinical outcome could be due to several factors, possibly including low bacterial loads and mild pathogenic capacities of the colonizing germs. In addition, it is not yet clear if certain types of CPE and/or certain types of carbapenemase could have a higher pathogenicity than others. Nevertheless the observation that none of the eight patients colonized by VIM-producing *C. freundii* had signs of clinical infection related to this MDR organism should not lead to the underestimation of the value of active surveillance measures, because any further spread to other patients might have resulted in more serious clinical effects.

In conclusion, our data confirm that in order to contain and control the spread of carbapenem-resistant *Enterobacteriaceae*, it is necessary to maintain and enforce all the infection control measures, among which a pivotal role is played by microbiological screening of potentially colonized patients.

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*Conflict of interest:* None declared.

## References

1. Cantón R, Akóva M, Carmeli Y, Giske CG, Glupczynski Y, Gniadkowski M, et al. Rapid evolution and spread of carbapenemases among *Enterobacteriaceae* in Europe. *Clin Microbiol Infect* 2012;**18**:413–31.
2. Gaibani P, Ambretti S, Berlingeri A, Gelsomino F, Bielli A, Landini MP, et al. Outbreak of NDM-1-producing *Enterobacteriaceae* in northern Italy, July to August 2011. *Euro Surveill* 2011;**16**:20027.
3. Gaibani P, Ambretti S, Berlingeri A, Cordovana M, Farruggia P, Panico M, et al. Rapid increase of carbapenemase-producing *Klebsiella pneumoniae* strains in a large Italian hospital: surveillance period 1 March–30 September 2010. *Euro Surveill* 2011;**16**. pii: 19800.
4. Ambretti S, Gaibani P, Caroli F, Miragliotta L, Sambri V. A carbapenem-resistant *Klebsiella pneumoniae* isolate harboring KPC-1 from Italy. *New Microbiol* 2010;**33**:281–2.
5. Koratzanis E, Souli M, Galani I, Chryssouli Z, Armaganidis A, Giamarellou H. Epidemiology and molecular characterisation of metallo-β-lactamase-producing *Enterobacteriaceae* in a university hospital intensive care unit in Greece. *Int J Antimicrob Agents* 2011;**38**:390–7.
6. Protonotariou E, Tsalidou M, Vitti D, Kalogeridis A, Sofianou D. First identification of VIM-1-producing *Citrobacter freundii* in Greece. *Int J Antimicrob Agents* 2008;**32**:460–1.
7. Rasheed JK, Biddle JW, Anderson KF, Washer L, Chenoweth C, Perrin J, et al. Detection of the *Klebsiella pneumoniae* carbapenemase type 2 carbapenem-hydrolyzing enzyme in clinical isolates of *Citrobacter freundii* and *K. oxytoca* carrying a common plasmid. *J Clin Microbiol* 2008;**46**: 2066–9.
8. Aschbacher R, Pagani L, Doumith M, Pike R, Woodford N, Spoladore G, et al. Metallo-β-lactamases among *Enterobacteriaceae* from routine samples in an Italian tertiary-care hospital and long-term care facilities during 2008. *Clin Microbiol Infect* 2011;**17**:181–9.
9. European Committee on Antimicrobial Susceptibility Testing (EUCAST). 2012. Breakpoint tables for interpretation of MICs and zone diameters Version 2.0, valid from 2012-01-01. [accessed 15 Aug 2012] Available at: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_2.0_120221.pdf.
10. Ambretti S, Gaibani P, Berlingeri A, Cordovana M, Tamburini MT, Bua G, et al. Evaluation of phenotypic and genotypic approaches for the detection of class A and class B carbapenemases in *Enterobacteriaceae*. Microb Drug Resist 2012; in press.
11. Mendes RE, Kiyota KA, Monteiro J, Castanheira M, Andrade SS, Gales AC, et al. Rapid detection and identification of metallo-beta-lactamase-encoding genes by multiplex real-time PCR assay and melt curve analysis. *J Clin Microbiol* 2007;**45**:544–7.
12. Poirel L, Naas T, Nicolas D, Collet L, Bellais S, Cavallo JD, et al. Characterization of VIM-2, a carbapenem-hydrolyzing metallo-beta-lactamase and its plasmid- and integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France. *Antimicrob Agents Chemother* 2000;**44**: 891–7.
13. Bai L, Xia S, Lan R, Liu L, Ye C, Wang Y, et al. Isolation and characterization of cytotoxic, aggregative *Citrobacter freundii*. *PLoS One* 2012;**7**:e33054.
14. Agenzia sanitaria e sociale regionale - Regione Emilia Romagna. Indicazioni pratiche e protocolli operativi per la diagnosi,la sorveglianza e il controllo degli enterobatteri produttori di carbapenemasi nelle strutture sanitarie e socio-sanitarie. [Practical indication and operative protocols for diagnosis, surveillance, and control of carbapenem-resistant Enterobacteriaceae in healthcare facilities]. Bologna: Regione Emilia Romagna; July 2012. Italian. Available from: http://asr.regione.emilia-romagna.it/wcm/asr/aree_di_programma/rischioinfettivo/gr_ric/pr_antibres/pubblicazioni/carbapenemasi_generale/link/carbapenemasi-generale.pdf.